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MAJOR ARTICLE

Molecular Epidemiology of Adenovirus Type 4 Infections in US Military Recruits in the Postvaccination Era (1997–2003)

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Background. Military recruits are at a higher risk of respiratory infection than their civilian counterparts. Continuous outbreaks of adenovirus (Ad)–associated acute respiratory disease were documented among US trainees before the implementation of serotype 4 (Ad4) and serotype 7 vaccines in 1971. The discontinuation of Ad vaccination programs in 1999 precipitated the reemergence of Ad in training sites, with Ad4 accounting for 98% of all diagnosed cases.

Methods. A total of 724 Ad4 strains isolated from recruits presenting with febrile respiratory illness at 8 training sites nationwide between 1997 and 2003 were genome typed by restriction enzyme analysis.

Results. Seven genome types were identified, all of which were distinct from the prototype Ad4p and the vaccine type 4p1. Results showed very different, and often stable, genome type distributions at different geographic sites, despite the homogeneity of the recruit source population.

Conclusions. The data support the hypothesis that reservoirs for Ad outbreaks are within recruit training sites or in their immediate environments, not in the incoming recruit population. Molecular characterization beyond serotype is critical to understanding the transmission dynamics of Ad infection in these unique susceptible populations and to the implementation of effective prevention approaches.

Human adenoviruses (Ads) are important ubiquitous pathogens responsible for a variety of illnesses, including acute respiratory disease (ARD), pharyngoconjunctivitis, and gastroenteritis. A total of 51 serotypes have been identified since the first discovery of these viruses in 1954 [1]. These 51 types are classified within 6 species (A–F) on the basis of the relative sequence homology of their genomic DNA, tissue tropism, fiber protein characteristics, and other biological properties [2].

The serotypes most frequently associated with respiratory infection are classified within subspecies B1 (Ad3, Ad7, and Ad21), species C (Ad1, Ad2, Ad5, and Ad6) and species E (Ad4). Some of these serotypes, like Ad3, Ad7, and particularly Ad4, are also frequent causative agents of epidemic conjunctivitis [3–5]. Clinical manifestations of Ad-associated respiratory disease range from febrile respiratory illness (FRI) to pharyngitis and pneumonia and may depend on the Ad serotype, the age of the patient, and other factors.

For reasons that are not totally clear, military recruits are at a higher risk of respiratory infection than their civilian counterparts [6, 7]. Stress and crowding are important factors that could facilitate transmission and

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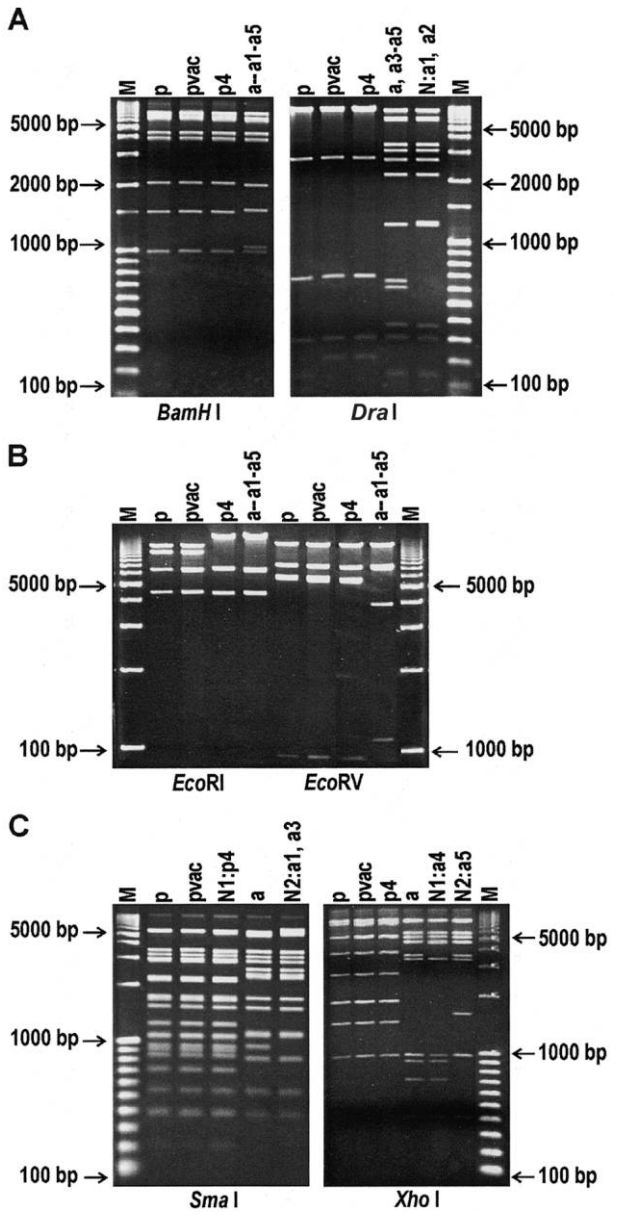

Figure 1. *A,* Restriction enzyme analysis of adenovirus 4 (Ad4) strains with *Bam*HI and *Dra*I. *B,* Restriction enzyme analysis of Ad4 strains with *Eco*RI and *Eco*RV. C, Restriction enzyme analysis of Ad4 strains with *Sma*I and *Xho*I. *Lane M,* molecular-weight markers (1 kb + 100 bp ladders; Bio-Rad); *lane p,* prototype strain of Ad4 (RI-67); 1, 2, etc., 4a variants; a, a distinct *Bam*HI restriction profile; N, novel restriction profiles not previously reported for these enzymes; p, prototype-like restriction profile; pvac, vaccination strain.

increase susceptibility [8, 9]. Continuous outbreaks of Ad-associated respiratory disease were documented among US military trainees and some deployed troops [10] before the implementation of the live enteric-coated Ad4 and Ad7 vaccines in 1971 [11, 12] and since 1996, when the production of the licensed vaccine formulation ceased. Similar associations between Ad and high rates of ARD have been seen in military trainee populations in other countries [13–16]. Since the loss of the vaccine, FRI rates have more than doubled among US military recruits, and a true reemergence of Ad infections has been noted. In the absence of the vaccine, it has been estimated that 10%–20% of all military recruits become ill with a febrile Ad infection during training [17, 18]. Large outbreaks of Ad-associated illness have been reported in recruit training facilities nationwide, with serotypes 4 and 7 being associated with the most recently described disease epidemics [19–21]. Seroprevalence studies conducted on incoming (pretraining) recruits demonstrated that >70% of all tested individuals lacked protective antibodies against Ad4 or Ad7 [17, 22]. Thousands of isolates have been obtained over the past decade that have documented the role of Ad4, the only known serotype of species E, as the leading causative agent of FRI in military trainees since 1996 [20, 21, 23–25]. Several fatalities associated with Ad infection of young adults have been recorded, including a well-documented case of Ad4-associated nonbacterial pneumonia [26–28].

The use of restriction enzyme analysis in the characterization of adenoviral genomes has revealed significant intraserotypic genetic variability for all serotypes examined to date. Restriction analysis of field isolates of Ad4 recovered worldwide has shown the existence of a variety of genome types (also known as DNA variants) belonging to 2 major clusters of homology—4p-like and 4a-like [29–32]. Most of the published molecular epidemiological studies of Ad4 infection have used restriction enzyme analyses of whole genomic DNA to characterize strains associated with conjunctivitis [33–35]. Very few have reported the genome type characterization of strains recovered from cases of respiratory infection [36, 37], possibly because of the

Table 1. Restriction enzyme analysis of adenovirus 4 (Ad4) genome types associated with outbreaks of febrile respiratory illness in US military recruits between 1997 and 2003.

	Digestion profile					
Genome type	*Bam*HI	*Dra*I	*Eco*RI	*Eco*RV	*Sma*I	*Xho*I
Ad4p4	p	p	a/p3	p	N1	p
Ad4a	a	a	a	a	a	a
Ad4a1	a	N	a	a	N2	a
Ad4a2	a	N	a	a	a	a
Ad4a3	a	a	a	a	N2	a
Ad4a4	a	a	a	a	a	N1
Ad4a5	a	a	a	a	a	N2
Ad4p	p	p	p	p	p	p
Ad4vac	p	p	p	p	p1	p

NOTE. Digestion profiles with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Sma*I, and *Xho*I were designated in accordance with the guidelines and reference patterns of Li and Wadell [30]. Novel restriction profiles for a given endonuclease were designated N, or N1, N2, etc., when >1 novel profile was identified. The digestion profiles of the prototype and vaccine strains of Ad4 are listed for comparison.

Table 2. Location of *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Sma*I, and *Xho*I restriction sites in the genomes of adenovirus 4 (Ad4) genome types 4p, 4pvac, 4p4, 4a, and 4a1–5.

Restriction enzyme, genome type	Sites, no.	Site location, nt
*Bam*HI		
p, pvac, p4	7	2041; 3538; 12,387; 16,801; 23,892; 24,885; 28,799
a, a1–a5	8	2005; 3538; 12,316; 16,737; 23,810; 28,685; 28,803; 29,791
*Dra*I		
p, pvac, p4	5	204; 484; 1164; 3913; 21,725
a, a3–a5	15[a]	59; 203; 483; 1113;[a] 1728; 3906; 4274; 18,518; 21,647; 25,104; 27,791; 28,997; 35,652; 35,763; 35,907
a1–a2	14	59; 203; 484; 1729; 3911; 4278; 18,520; 21,649; 26,106; 27,793; 28,999; 35,653; 35,664; 35,905
*Eco*RI		
p, pvac	3	10,655; 25,361; 29,702
p4, a, a1–a5	2	25,291; 29,562
*Eco*RV		
p, pvac, p4	4	7178; 23,959; 29,619; 35,053
a, a1–a5	4	7179; 23,887; 31,012; 34,871
*Sma*I		
p	20	888; 2543; 4058; 4599; 5686; 9170; 9395; 12,430; 12,833; 13,552; 14,514; 16,687; 19,928; 21,177; 22,234; 25,188; 26,041; 26,432; 28,142; 33,780
pvac	19	888; 2543; 4058; 4599; 5686; 9395; 12,430; 12,833; 13,552; 14,514; 16,687; 19,928; 21,177; 22,234; 25,188; 26,044; 26,435; 28,145; 33,784
p4	20	887; 2544; 4060; 4601; 5688; 9172; 9397; 12,412; 12,815; 13,534; 14,496; 16,669; 19,898; 21,147; 22,204; 25,158; 26,014; 26,405; 28,115; 33,707
a, a2, a4–a5	15	2543; 4059; 4600; 5687; 9171; 12,369; 13,497; 14,441; 19,859; 22,168; 25,116; 25,948; 26,338; 28,048; 33,598
a1, a3	14	2543; 4059; 4600; 5687; 9171; 12,369; 13,497; 19,859; 22,168; 25,116; 25,948; 26,338; 28,048; 33,598
*Xho*I		
p, pvac, p4	9	3708; 3772; 5625; 7488; 10,151; 15,405; 16,371; 25,034; 34,554
a, a1–a3	10	5626; 6598; 10,152; 15,351; 16,317; 26,101; 26,499; 29,826; 34,372; 35,266
a4	9	5626; 10,152; 15,351; 16,317; 26,101; 26,499; 29,826; 34,372; 35,266
a5	9	5626; 6598; 10,152; 15,351; 16,317; 26,101; 26,499; 29,826; 34,372

NOTE. Restriction sites were first identified on available complete genome sequences for Ad4p (strain RI-67, GenBank accession no. AY594253), Ad4pvac (accession nos. AY594254 and AY458656), and Ad4 field strains NHRC 90339 (genome type 4p4; accession no. EF371058), NHRC 42606 (genome type 4a2; accession no. AY599835), and NHRC 3 (genome type 4a1; accession no. AY599837), using SeqBuilder software (Lasergene suite, version 1.0; DNASTAR). Information was extrapolated to genome types 4a and 4a variants a3–a5 based on the presence or absence of comigrating restriction fragments.

[a] Restriction site and location were estimated and are subject to verification by whole-genome sequencing.

infrequent association of Ad4 with respiratory disease among civilians. Despite the leading role of Ad4 in the etiology of respiratory illness in military training facilities, little is known about the magnitude of genetic variability among Ad4 strains circulating in the United States since the original identification of Ad4 [1, 38]. Recent publications have suggested that a new strain of Ad4 has recently emerged among military trainees [39–42], but the new strain has not been genome typed, nor has its distribution or prevalence been assessed. In the absence of genome type–specific surveillance, the very high prevalence of Ad4 has prevented any meaningful analysis of strain distribution and strain-specific transmission in this highly affected population.

The impact of genetic variation on Ad fitness and virulence is not known, but it is extremely likely that different Ad genome types display phenotypic differences that may involve their antigenic reactivity as well as their pathogenic potential. A recent retrospective study of a few Ad4 and Ad7 strains isolated between 1953 and 1997 showed evidence of genetic variability in hypervariable regions of the hexon gene resulting in detectable differences in neutralization titers [42]. Although these studies were conducted on a relatively small number of viral strains,

Table 3. Molecular size of DNA fragments of adenovirus 4 (Ad4) genome types generated by digestion with 6 endonucleases.

Restriction enzyme, genome type	Fragment size, bp
*Bam*HI	
4p/pvac/p4[a]	8841; 7212; 7090; 4414; 3909; 2040; 1495; 993
4a, a1–a5[a]	8782; 7072; 6244; 4421; 3882; 2004; 1529; 1037; 993
*Dra*I	
4p/pvac/p4[a]	17,773; 14,435; 2746; 682; 280; 203
4a, a3–a5	14,242; 6654; 3457; 3129; 2687; 2182; 1206; 630; 610; 367; 281; 144; 144; 100; 59; 57
4a1, a2 N[a]	14242; 6654; 3457; 3129; 2687; 2182; 1245; 1206; 367; 281; 144; 144; 100; 59; 57
*Eco*RI	
4p/pvac[a]	14,706; 10,655; 6288; 4341
4p4/4a, a1–a5[a]	25,289; 6403; 4272
*Eco*RV	
4p/pvac[a]	16,781; 7178; 5660; 5464; 941
4a, a1–a5[a]	16,708; 7179; 7125; 3859; 1093
*Sma*I	
4p[a]	5638; 3484; 3241; 3098; 3035; 2954; 2173; 1710; 1655; 1515; 1249; 1087; 1057; 962; 888; 856; 719; 541; 403; 391; 225
4pvac[a,b]	5639; 3709; 3241; 3098; 3035; 2954; 2173; 1710; 1655; 1515; 1249; 1087; 1057; 962; 888; 856; 719; 541; 403; 391
4p4 N1[c]	5592; 3484; 3229; 3096; 3015; 2954; 2173; 1710; 1657; 1516; 1249; 1087; 1057; 962; 887; 856; 719; 541; 403; 391; 225
4a	5550; 5400; 3484; 3198; 2948; 2547; 2366; 2309; 1710; 1516; 1128; 1087; 963; 832; 541; 390
4a1, a3 N2[a]	6362; 5550; 3484; 3198; 2948; 2543; 2366; 2309; 1710; 1516; 1128; 1087; 832; 541; 390
*Xho*I	
4p/pvac/p4[a]	9524; 8663; 5254; 3708; 2663; 1863; 1853; 1436; 966; 64
4a, a1–a3[a]	9785; 5630; 5199; 4546; 3326; 972; 966; 894; 698; 398
4a4 N1[c]	9785; 5630; 5199; 4546; 4526;, 3326; 966; 894; 698; 398
4a5 N2[c]	9785; 5630; 5199; 4546; 3326; 972; 966; 398

[a] Fragment sizes were calculated from sequence data available from GenBank for Ad4p RI-67 (accession no. AY594253), Ad4pvac (accession no. AY594254), and Ad4 field strains NHRC 90339 (genome type 4p4; accession no. EF371058), NHRC 42606 (genome type 4a2; accession no. AY599835), and NHRC 3 (genome type 4a1; accession no. AY599837), using SeqBuilder 1.0 software.

[b] Our results revealed a discrepancy with the profile predicted from the vaccine strain CL68578 genomic sequence AY458656.

[c] Fragment sizes were estimated by mobility analysis in horizontal agarose gels. N, N1, and N2 designate the novel restriction profiles shown in figure 1.

they clearly demonstrated the occurrence of an antigenic drift for Ad4 and raised questions related to the long-term adequacy of protection conferred by the present Ad4-Ad7 vaccine design. In view of these limited but intriguing findings, the molecular characterization of viral isolates during surveillance of adenovirus infections in military settings is needed to understand Ad infection and transmission dynamics in this highly susceptible population and to assess the efficacy of the present Ad vaccination program renewal efforts within the Department of Defense (DoD). In the present article, we report the characterization by restriction enzyme analysis of 724 Ad4 strains isolated from cases of FRI in military personnel in 8 military training sites in the United States over the course of a 7-year period after the 1996 discontinuation of Ad vaccine production.

MATERIALS AND METHODS

Surveillance of adenovirus infection among US military recruits. The Respiratory Disease Laboratory at the Naval Health Research Center (NHRC) conducts active surveillance for respiratory pathogens as the US Navy node for the DoD Global Emerging Infections Surveillance and Response System (http://www.geis.fhp.osd.mil/). Beginning in October 1996, surveillance for Ad infection was established and at present includes 8 military training camps throughout the United States. Collaborators and NHRC staff at each site monitor their trainee population for FRI symptoms. Clinical specimens are routinely obtained from a subset of recruits who seek medical care. These samples are then sent to NHRC to be tested for Ad, influenza A/B, parainfluenza 1–3, and respiratory syncytial virus.

Origin of virus strains. Genome typing work was performed on a collection of 724 Ad4 strains isolated from the pharyngeal swabs of military trainees presenting with symptoms

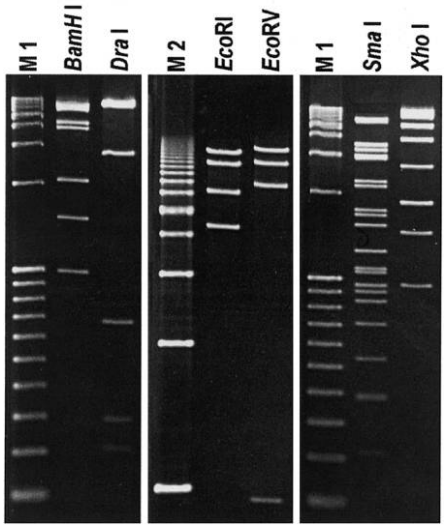

Figure 2. Restriction enzyme analysis of adenovirus 4 (Ad4) strains isolated in the United States before implementation of vaccination programs. Seven Ad4 strains isolated in the United States in 1966, 1968, and 1971 were characterized by digestion with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Sma*I, and *Xho*I. Identical profiles were found for all strains with the 6 endonucleases.

Table 4. Origin of adenovirus 4 strains isolated in the United States before the implementation of a vaccination program.

Strain ID	Date of isolation	Site	Genome type
RU 2528	May 1966	Cape May, NJ	4p
RU 2533	April 1966	Cape May, NJ	4p
RU 2541	May 1966	Cape May, NJ	4p
RU 4445	May 1968	NAMRU, IL	4p
RU 7441	March 1971	North Dakota	4p
RU 7442	March 1971	North Dakota	4p
RU 7872	May 1971	Mayo Clinic, MN	4p

NOTE. NAMRU, Naval Medical Research Unit.

of respiratory disease between 1997 and 2003 at 8 training sites in the Unites States: Fort Jackson, South Carolina; Fort Benning, Georgia; Fort Leonard Wood, Missouri; Naval Recruit Training Command, Great Lakes, Illinois; Lackland Air Force Base, San Antonio, Texas; Coast Guard Training Center, Cape May, New Jersey; Marine Corps Recruit Depot, Parris Island, South Carolina; and Marine Corps Recruit Depot, San Diego, California. The 724 Ad4 isolates were chosen to represent the overall population as evenly as possible, both temporally and spatially (i.e., samples were selected to include all sites and all times, rather than being a random subset of all samples collected). Because of this, apparent temporal trends in sample size do not necessarily represent changes in rates at the sites. All virus samples were initially isolated in A549 cell monolayers and confirmed to be Ads by immunofluorescence in the College of American Pathologists–certified NHRC diagnostic microbiology laboratory. The Ad isolates analyzed in this study were identified as Ad4 by a microneutralization assay [43], polymerase chain reaction (PCR) [44], or both. Serotyped isolates were then stored at −80°C for further analysis.

Case definition. A case of FRI/ARD was defined as a recruit presenting for medical care and meeting the following 2 criteria: fever (oral temperature ⩾38°C/100.5°F) and a respiratory symptom (cough or sore throat).

Viral DNA purification and restriction enzyme analysis. Ad isolates were shipped in dry ice to the Lovelace Respiratory Research Institute for further characterization. All isolates were passed once onto monolayers of A549 cells in 25-cm^2 flasks and subsequently amplified in monolayers of A549 cells in 75-cm^2 flasks for viral DNA extraction. When an extensive cytopathic effect was evident, intracellular viral DNA was isolated from infected cells using the method developed by Shinagawa et al. [45], with modifications. For genome type identification, 1 μg of viral DNA was initially digested with restriction endonuclease *Bam*HI in accordance with the manufacturer's recommendations (Promega). Viral DNA was further characterized by digestion with *Dra*I, *Eco*RI, *Eco*RV, *Xho*I, and *Sma*I. DNA fragments were separated by horizontal gel electrophoresis in 0.8% or 1.2% agarose gels run in 1× Tris borate–EDTA buffer (0.09 mol/L Tris borate and 0.002 mol/L EDTA [pH 8]). Restriction profiles were visualized by UV transillumination at 303 nm after staining with ethidium bromide and were photographed in a Gel Doc imager (Bio-Rad).

Restriction sites were mapped and fragment sizes calculated using available whole genome sequence data for the prototype strain RI-67 (Ad4p, genome type 4p; GenBank accession number AY594253), the vaccine strain CL68578 (Ad4pvac, genome type 4p1; accession numbers AY594254 and AY458656), and 3 Ad4 field isolates from recruit populations genome typed in the present study—NHRC 90339, NHRC 42606, and NHRC 3 (GenBank accession numbers EF371058, AY599835, and AY599837, respectively) using SeqBuilder software (Lasergene suite version 1.0; DNAStar). Maps for novel genome types were inferred from these data by identification of comigrating restriction fragments after comparison of their restriction profiles with those of the prototype, vaccine, and field strains.

Genome type denomination. Genome types were denominated in using the system developed by Li and Wadell [46] through comparison of the generated restriction patterns with published profiles. Genome types or DNA variants were initially discriminated on the basis of their distinct profile of *Bam*HI restriction fragments and designated *p* for the prototype strain, and *a, b, c,* and so forth, for subsequently identified variants. Additional restriction enzymes discriminate subtypes p1, p2, a1, a2, and so forth. In the present article, novel restriction profiles not previously reported for a given endonuclease were designated N or N1, N2, and so forth, when >1 novel profile was identified.

PCR confirmation of coinfections. The only 2 detected cases of apparent coinfections between a p-like genome type and an a-like genome type were confirmed by PCR using an E1A-targeted primer pair amplifying a region in which the Ad4a and Ad4p amplicons differ by 33 bp (Ad4a yields 222 bp, whereas Ad4p yields 255 bp). Primers used were AdEF (5′-CTGCACGATTTGTATGATCTGG-3′) and AdER (5′-CCTGCTCGTICTCATCATCG-3′). Samples were extracted using the Qiagen 96 DNA Blood Kit (Qiagen) in accordance with the manufacturer's instructions, and reactions were performed using the Qiagen Multiplex Kit (Qiagen) in accordance with the manufacturer's recommendations. Reactions were cycled in a Bio-Rad iCycler using standard parameters with a 58°C annealing temperature.

RESULTS

Contribution of Ad4 to total Ad morbidity. Data from continuous surveillance have shown that, during 1996, the last year of full Ad4/Ad7 vaccine administration, Ad4 infections accounted for only 4% of the total Ad morbidity among US military recruits [39]. The proportion of Ad-associated mor-

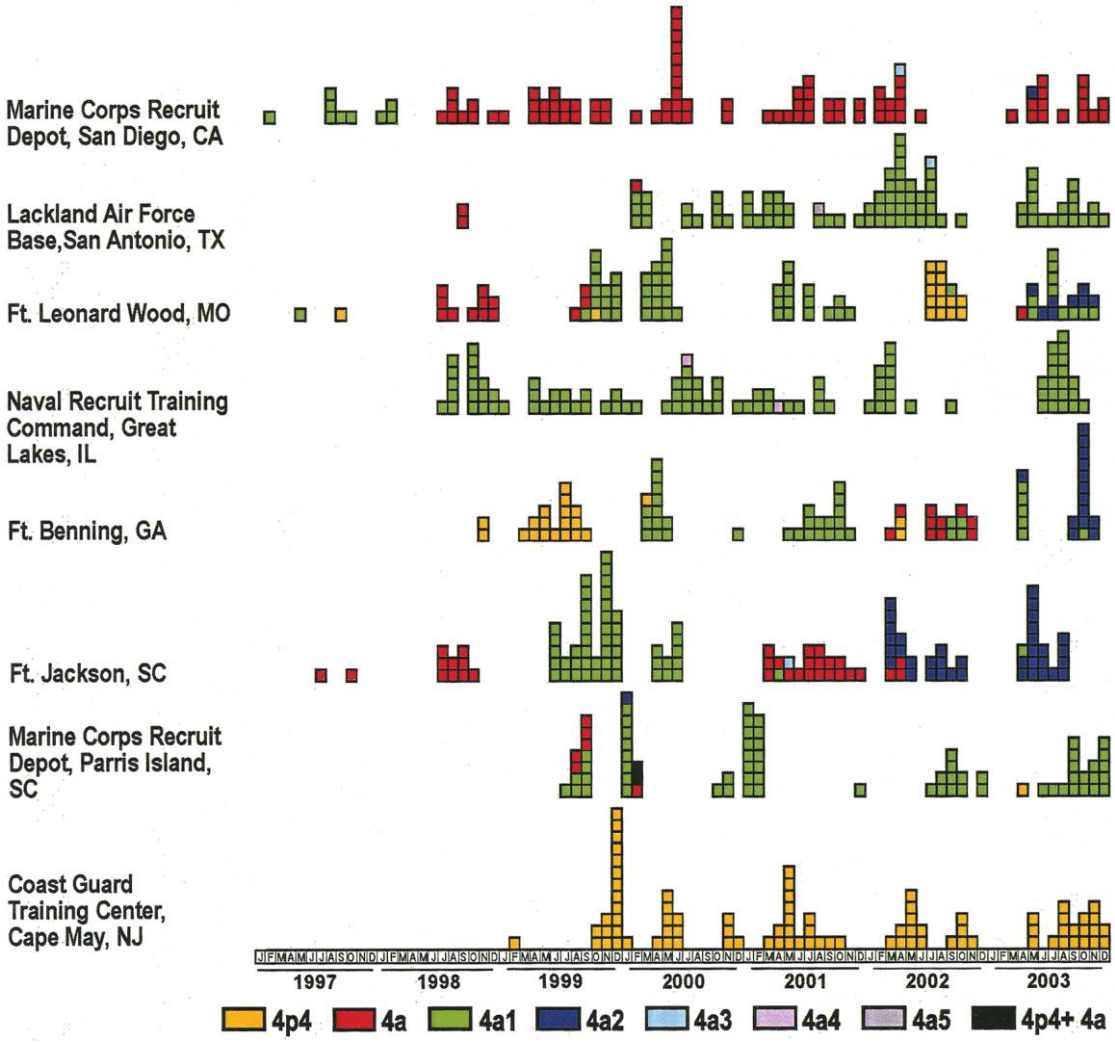

Figure 3. Geographic and temporal distribution of adenovirus 4 genome types in United States military training sites (1997–2003)

bidity attributable to Ad4 rapidly increased to 58% in 1997 and to 73% in 1998 as vaccine stocks were progressively consumed. In 1999, after complete depletion of the vaccine supplies, Ad4 became responsible for 98% of all diagnosed cases of Ad infection and remained at this proportion or higher for the remainder of the analysis period, with negligible representation from other serotypes [39].

Genome typing of military Ad4 strains isolated between 1997 and 2003. Characterization of viral DNA using a panel of 6 endonucleases (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Xho*I, and *Sma*I) revealed the presence of 7 distinct genome types belonging to 2 major clusters—4p-like and 4a-like—as defined by their *Bam*HI profiles. None of the identified DNA variants corresponded to the Ad4 prototype strain (4p) or to the Ad4 vaccine strain (described by Li and Wadell [30] as corresponding to genome type 4p1). Instead, all 4p-like isolates corresponded to a genome type not previously described, 4p4 that exhibited distinctive *Eco*RI profiles identical to those described for genome type 4p3 but unique *Sma*I restriction patterns. Restriction analysis of 4a-like genomes with *Dra*I, *Sma*I, and *Xho*I allowed the identification of novel patterns and, from those patterns, the discrimination of 6 different variants or subtypes: 4a, 4a1, 4a2, 4a3, 4a4, and 4a5. Of these 6 variants, only 4a had been described in the literature for North American Ad4 strains [30]. Restriction patterns for each of the identified genome types with all 6 enzymes are shown in figure 1*A*, 1*B*, and 1*C* in comparison with the prototype strain (p) and the vaccine strain (pvac) of Ad4 and are summarized in table 1. The restriction-site maps and estimated restriction-fragment sizes for each endonuclease and each individual genome type are shown in tables 2 and 3.

Genome type analysis of Ad4 strains isolated in the United States before the implementation of vaccine protocols. Seven Ad4 strains isolated in the United States in 1966, 1968, and 1971 were provided for the study by Dr. D. Erdman (Centers for Disease Control and Prevention, Atlanta, GA). These strains

were characterized using the same panel of 6 endonucleases as those described above. All 7 strains yielded identical prototype-like profiles after digestion with *Bam*HI and identical profiles after digestion with *Dra*I, *Eco*RI, *Eco*RV, *Sma*I, and *Xho*I (figure 2 and table 4) indistinguishable from the patterns generated by digestion of prototype strain RI-67 DNA; they were therefore identified as corresponding to genome type 4p.

Geographic and temporal distribution of Ad4 genome types. The analysis of the temporal and geographic occurrence of the 7 identified genomic variants of Ad4 revealed a training site–specific pattern of circulation as shown in figure 3. Genome type Ad4a1 was the most frequently isolated and most widely distributed DNA variant, accounting for 391 of 724 isolates examined and circulating in 7 of 8 training sites for variable periods of time since 1997. Two cases of coinfection with DNA variants Ad4p4 and Ad4a were identified in specimens collected in Parris Island, South Carolina, in 2000, as revealed by the presence of overlapping patterns in the digests of the extracted viral DNA (figure 4*A*). Restriction analysis of these samples with *Eco*RV, *Sma*I, and *Xho*I clearly showed the coexistence in the digests of fragments corresponding to both genome type 4p4 and genome type 4a. These coinfections were confirmed by PCR (figure 4*B*) using a pair of primers targeting a region of the E1A gene in which 4a-like DNA variants and 4p-like DNA variants differ by a 30-bp insertion/deletion.

DISCUSSION

Ad morbidity among military personnel has been increasing since 1994, when vaccine production delays resulted in shortages, and markedly since 1996, when the sole manufacturer of the licensed vaccine permanently discontinued production. Before vaccination was started in 1971, Ad4 and Ad7 were the major causes of ARD in basic trainees [12, 47]. A true re-emergence of Ad4 has been noticed in recent outbreaks of respiratory illness among recruits, with isolation rates reaching 90% and delays in training frequently being required [17, 18]. The relative contribution of Ad4 to total adenovirus morbidity has steadily increased since 1996, reaching 98% of all serotyped Ad isolates in 1999 and staying at these rates through 2003 [39]. The resulting disruption of training has compromised predeployment troop readiness, challenged medical resources, and generated significant financial burdens to training commands. Until the restoration of Ad4/Ad7 vaccine programs, which are estimated to begin no earlier than 2009 [17], epidemics of Ad-associated respiratory illness should be anticipated in recruit training camps.

The body of molecular analyses conducted in the present study offers important insights into the epidemiology of Ad4 infections in recruit training sites. Our data clearly show the circulation in military recruit populations over the past decade of Ad4 strains corresponding to 2 major clusters of genomic homology—4p-like and 4a-like—and representing DNA variants easily distinguishable from the vaccine and prototype strains by restriction enzyme analysis. Ad4 strains corresponding to Ad4p-like and Ad4a-like genome types have been previously isolated in association with both respiratory disease and conjunctivitis worldwide [29, 32, 36, 48], but no particular associations of individual genomic variants with specific clinical presentations have been demonstrated. The disappearance of vaccine selective pressure is likely to be one of the major reasons for the emergence of new Ad4 genome types and for changes in the relative prevalence of existing DNA variants in the military recruit setting. Limited data, including those generated in the present study, indicate that 4p-like genome types were the most prevalent variants in the United States during the late 1960s to early 1980s [30, 31]. Interestingly, there is some evi-

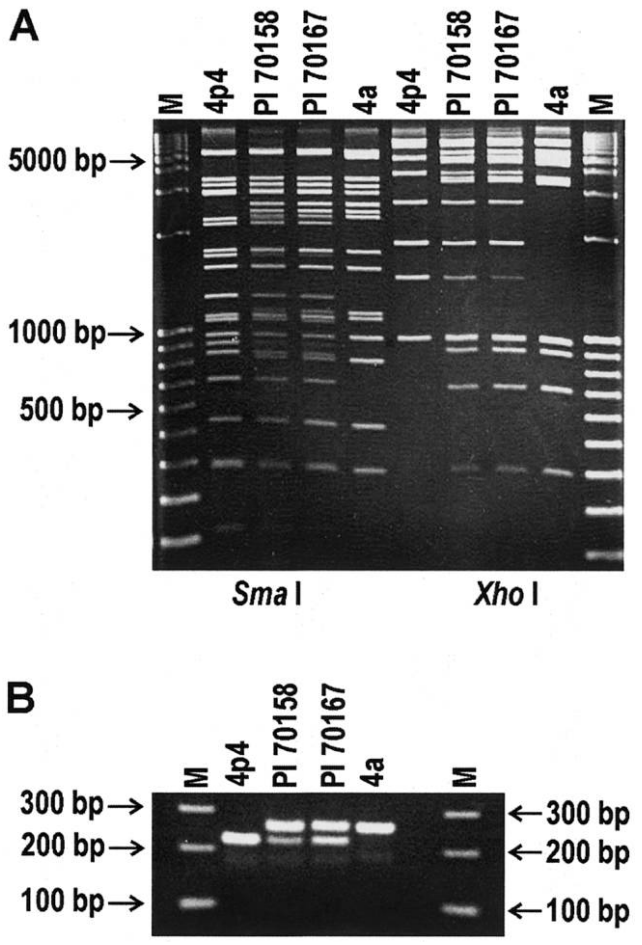

Figure 4. *A,* Restriction enzyme analysis of adenovirus 4 (Ad4) isolates representing coinfections with 2 genome types. *B,* Polymerase chain reaction (PCR) analysis of Ad4 isolates representing coinfections with 2 genome types. Amplicons represent the products of a monoplex PCR targeting a length-polymorphic segment of the E1A region. Single bands represent infections of Ad4a or Ad4p alone, whereas double bands confirm the coinfections seen by restriction enzyme analysis. M, molecular-weight marker.

dence documenting the circulation in the country of strains corresponding to genome type 4a in the late 1970s [30].

A recent study implicating the environment in the chain of transmission of Ad infections among trainees [18] demonstrated the complete lack of Ad4 among recruits when they initially arrived for training and evidence of widespread transmission of a given strain of Ad during training. Additionally, the same study showed that Ad4 can be found ubiquitously in the recruits' physical environment, often on surfaces such as footlockers, rifles, restroom fixtures, and pillows, which suggests that Ad4 is highly resistant to environmental degradation and may use the physical environment as a reservoir. The site-specific nature of the genome type distributions found in the present study may be best supported by our findings at 2 sites (New Jersey and Illinois), where very different DNA variants were retained for the entire study period, which supports the hypothesis that there are local reservoirs of virus acting as consistent sources for the observed outbreaks. All training sites displayed uncorrelated and unique temporal genome type distributions, despite the fact that all camps receive recruits from the same source population. This appears to exclude incoming recruits as a likely primary source of epidemiologically relevant adenovirus infections.

The temporal distribution of genome types over the 7-year period covered by this study showed a strong tendency toward homogeneity at specific times within single training facilities, with only occasional cocirculation of multiple types. Transitions of dominance between genome types tended to be very sharp. These dynamics are suggestive of strong selective pressures driving replacement events. However, transitions from 1 dominant DNA variant to another in a given site were of a generally random nature. On some occasions, Ad4a-like genome types appeared to replace Ad4p4, whereas, on other occasions, the opposite substitution was observed. This pattern suggests selective dynamics driven more by local herd immunity to recently dominant strains rather than by any inherent selective advantage of one genome type relative to another, although possible differences in fitness and transmissibility between DNA variants should not be completely ruled out. Antigenic differences between genome types may explain the observed temporal occurrence of genome types in a given site and should be evaluated in the context of the ongoing clinical trials for the reimplementation of Ad vaccines. The identification of 2 cases of coinfection with Ad4p4 and Ad4a is noteworthy, because homologous recombination, which has been shown to occur in nature between closely related genomes, is a well-acknowledged mechanism of Ad evolution [49, 50].

While this study was being conducted at NHRC and Lovelace Respiratory Research Institute, the full genome sequence of Ad4a was determined at the laboratory of H.-S. Huong at Walter Reed Army Institute of Research (WRAIR). That work demonstrated the replacement of the inverted terminal repeat (ITR) in the prototypical Ad4p-like genomes with a species B–like ITR in the divergent Ad4a-like strains currently dominant at many recruit camps [40]. In an effort to assay the distribution of this mutation among adenovirus strains circulating in recruit training facilities, WRAIR obtained a collection from NHRC of Ad4 isolates representing the 8 sites under surveillance. Using a PCR targeted at the ITR polymorphism [40], WRAIR independently identified the consistent presence of a site-specific variant at the New Jersey training center (corresponding to the genome type Ad4p4 described in the present study), in contrast to the general dominance of ITR variant Ad4a (corresponding to the 4a-like genome types described in the present study) among the rest of the collection. This finding independently confirmed the site-restricted Ad genome type distributions in US recruit training facilities reported here.

The striking genetic diversity detected among Ad4 strains associated with recent outbreaks of febrile respiratory illness among military recruits and the intriguing site-specific pattern of circulation of the 7 Ad4 genome types identified in the present study may have strong implications for the design of long-term successful intervention strategies. Our results strongly suggest that the epidemiological characteristics and molecular dynamics of Ad4 infections in the military environment are complex and stratified at the genome type level of discrimination. This finding highlights the importance of continued genome type–specific surveillance and points to the epidemiological inadequacy of serological discrimination alone.

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Background: Military recruits are at a higher risk of respiratory infection than their civilian counterparts. Continuous outbreaks of adenovirus (Ad)-associated acute respiratory disease were documented among US trainees before the implementation of Ad4 and Ad7 vaccines in 1971. The discontinuation of Ad vaccination programs in 1996 precipitated the re-emergence of Ad in training sites, with serotype 4 (Ad4) accounting for 98% of all diagnosed cases.
Methods: Seven hundred and twenty-four (724) Ad4 strains isolated from recruits presenting with febrile respiratory illness at eight training sites nationwide between 1997 and 2003 were genome typed by restriction enzyme analysis.
Results: Seven genome types were identified, all distinct from the prototype Ad4p and the vaccine type 4p1. Results show very different, and often stable, genome type distributions at different geographic sites despite the diversity of the recruit source population.
Conclusions: The data support the hypothesis that reservoirs for Ad outbreaks are within recruit training sites or in their immediate environments, not in the incoming recruit population. Molecular characterization beyond serotype is critical to understanding the transmission dynamics of Ad infection in these unique susceptible populations, and to the implementation of effective prevention approaches.

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